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CK2 phosphorylation of eukaryotic translation initiation factor 5 potentiates cell cycle progression

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Casein kinase 2 (CK2) is a ubiquitous eukaryotic Ser/Thr protein kinase that plays an important role in cell cycle progression. Although its function in this process remains unclear, it is known to be required for the G_1 and G_2/M phase transitions in yeast. Here, we show that CK2 activity changes notably during cell cycle progression and is increased within 3 h of serum stimulation of quiescent cells. During the time period in which it exhibits high enzymatic activity, CK2 associates with and phosphorylates a key molecule for translation initiation, eukaryotic translation initiation factor (eIF) 5. Using MS, we show that Ser-389 and -390 of eIF5 are major sites of phosphorylation by CK2. This is confirmed using eIF5 mutants that lack CK2 sites; the phosphorylation levels of mutant eIF5 proteins are significantly reduced, relative to WT eIF5, both *in vitro* and *in vivo*. Expression of these mutants reveals that they have a dominant-negative effect on phosphorylation of endogenous eIF5, and that they perturb synchronous progression of cells through S to M phase, resulting in a significant reduction in growth rate. Furthermore, the formation of mature eIF5/eIF2/eIF3 complex is reduced in these cells, and, in fact, restricted diffusional motion of WT eIF5 was almost abolished in a GFP-tagged eIF5 mutant lacking CK2 phosphorylation sites, as measured by fluorescence correlation spectroscopy. These results suggest that CK2 may be involved in the regulation of cell cycle progression by associating with and phosphorylating a key molecule for translation initiation.

Casein kinase 2 (CK2) (1–4) is composed of two subunits, α or α' and β, which combine to form a native $\alpha_2\beta_2$ tetramer. Disruption of the catalytic subunits (α and α') is lethal in *Saccharomyces cerevisiae* (5) and disruption of the regulatory β subunit in mice leads to early embryonic lethality (6). CK2 phosphorylates a range of cellular targets in a variety of subcellular sites and appears to be highly pleiotropic; it is involved in many key biological functions, including growth and cell cycle control (7), signal transduction (3), circadian rhythms (8, 9), and gene expression (10, 11). CK2 is also a stress-activated kinase and might participate in the transduction of survival signals to avoid damage by mutagenic UV radiation (12, 13). An important role for CK2 in promoting cell proliferation and transformation has been indicated by several studies. In mammalian systems, its targeted overexpression in mice results in the development of T cell lymphoma and mammary tumorigenesis (5). Despite these findings, there is still much uncertainty regarding the activation of CK2 in response to stimuli (14). The mechanism by which it is regulated and its precise function in cell cycle progression and proliferation is still poorly understood.

CK2 activity and stability are believed to be regulated in part by holoenzyme formation via a self-assembly mechanism and by phosphorylation. Phosphorylation by $p34^{cdc2}$ of the catalytic α subunit at the C-terminal domain occurs in a cell cycle-dependent manner in mitotic cells. The regulatory β subunit is also autophosphorylated at four sites, including Ser-2, -3, -4, and -209, the latter being maximally phosphorylated in mitotic cells. So far, no clear effect of phosphorylation of CK2 on its activity has been demonstrated. Previously, we described a cell cycle-dependent interaction between CK2 and the adenomatous polyposis coli (APC) tumor suppressor protein and an inhibitory effect of APC on CK2 activity (15). This implies that CK2 activity can be controlled by interactions with regulatory molecules such as APC rather than by direct phosphorylation.

In this work, we demonstrate a significant increase in CK2 activity in cells induced to enter G_1 phase by growth factor stimulation. During this time period, CK2 associates with and phosphorylates eukaryotic translation initiation factor 5 (eIF5). We further identify the sites of eIF5 phosphorylation and show that eIF5 mutants that lack these phosphorylation sites attenuate cell cycle progression and proliferation. The formation of translation initiation complexes is also suppressed by the eIF5 mutants, resulting in suppression of expression of cell cycle regulators such as cyclin B_1. Our observations suggest that CK2 is involved in regulating translation and the cell cycle through the association and phosphorylation of eIF5, a key component in translation initiation.

Methods

Cell Culture. COS-7 cells, human embryonic kidney (HEK)293 cells, and normal human fetal lung fibroblasts TIG-7 were grown in DMEM supplemented with 10% FBS. For synchronization experiments, logarithmically growing cells were starved in 0.2% FBS for 48 h and then cultured in fresh media containing 10% FBS for an additional 16–20 h to obtain cell populations enriched in S phase. Alternatively, cells were arrested in prometaphase by adding 50 ng/ml nocodazole to the medium. For some experiments, cells were treated with apigenin (Sigma) at 80 μM for 2 h or with short interfering RNA (Upstate Biotechnology, Lake Placid, NY) to inhibit kinase activity.

Plasmids and Transfections. Full-length cDNAs for human CK2α and -β subunits were obtained as described (16). Human eIF5 cDNA was isolated from a cDNA library of human fetal fibroblast. Site-directed mutagenesis of eIF5 was performed to mutate Ser-389 and -390 to two Ala residues (M1) (M*n*, mutant *n* of eIF5) and to mutate Thr-207 and -208 to two Ala residues (M2). M3 with several mutations was generated by two rounds of mutagenesis by using M1 and M2. All constructs and mutations were confirmed by DNA sequencing (for further details, see *Supporting Methods*, which is published as supporting information on the PNAS web site).

Kinase Assays. CK2 activity was measured by p81-filter assay by using RRREEETEEE or RRRDDDSDDD as a substrate peptide (17). *In vitro* phosphorylation of eIF5 by CK2$\alpha\beta$ was assayed by incubating a reaction mixture consisting of 20 mM Hepes, pH 7.4/10 mM β-glycerophosphate/5 mM $MgCl_2$/10 μg/ml aprotinin/5 μg/ml leupeptin/1 mM PMSF/0.2 mM ATP/1 μCi [γ-^{32}P] ATP (1 Ci = 37 GBq), in the presence or absence of 10 ng/ml heparin at 30°C for 5 to 20 min. Phosphorylation reactions were

Abbreviations: APC, adenomatous polyposis coli; CK2, casein kinase 2; eIF5, eukaryotic translation initiation factor 5; TIG-7, normal human fetal lung fibroblasts; HEK, human embryonic kidney; M*n*, mutant *n* of eIF5.

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separated by SDS/PAGE (18), and ^{32}P incorporation was detected by autoradiography. For *in vivo* labeling, see *Supporting Methods*. Preparation of whole-cell lysates and the conditions for immunoblotting and immunoprecipation for cyclin B_1 kinase assays have been described (19).

Mass Spectrometric Analysis. Two eIF5 preparations were analyzed to identify phosphorylation sites. FLAG-tagged eIF5 was expressed in HEK293 cells, the cells stimulated with FBS for 6 h after serum starvation, and the eIF5 protein immunoprecipitated by anti-FLAG agarose to characterize the phosphorylation state *in vivo*. Recombinant eIF5 protein was phosphorylated *in vitro* by incubating with reconstituted CK2 holoenzyme. Both protein samples were separated by SDS/PAGE (12% acrylamide). Bands were excised and proteins digested with trypsin (Promega). Extracted peptides were analyzed by using QSTAR electrospray ionization TOF tandem MS (Applied Biosystems).

Analysis of Protein Complexes by Sucrose Density Centrifugation. HEK293 cells transfected with WT or M1 eIF5 were grown for 48 h and then treated with cycloheximide for 30 min before harvesting. Lysates were clarified by centrifugation at 23,000 × *g* for 30 min at 4°C to obtain the supernatant fractions with protein concentrations between 2 and 4 mg/ml (20). The sample was loaded on a 5–40% sucrose gradient prepared in a buffer containing 20 mM Tris·HCl, pH 7.5; 100 mM KCl; and 1 mM $MgCl_2$, and centrifuged at 250,000 × *g* (SW55Ti, Beckman) for 4.5 h at 4°C. The gradient was separated into 15 fractions of 0.2 ml, and the absorption of each fraction at 254 nm was monitored. Each fraction was used for eIF5 immunoprecipitation.

Analysis of Protein Dynamics in Living Cells. Introduction of GFP-fused eIF5 expression plasmids by using siliconized glass microbeads into cells was described previously (21). For fluorescence correlation spectroscopy (FCS) analysis, a ConfoCor2 instrument (Zeiss) was used and analysis carried out as described (22).

Results

Activation of CK2 in Response to FBS Stimuli. To investigate the control of CK2 activity and expression in relation to cell cycle progression, we examined changes in its activity in response to serum stimulation using TIG-7 cells synchronized by serum starvation. Expression of cyclin B_1 was monitored to verify cell synchronization (Fig. 1*B*). Cell lysates were prepared from unstimulated cells (G_0 phase in the cell cycle) and cells stimulated with 10% FBS for varying time periods. CK2 activity was enhanced in a time-dependent manner after the stimulation of quiescent cells when the synthetic peptide RRREEETEEE was used as substrate, reaching the highest level in the first 3 h and decreasing thereafter as cells progressed toward the G_2/M phase (Fig. 1*A*). In contrast, no significant alterations in CK2 activity were measured when using the synthetic peptide RRRDDDSDDD as a substrate. There were no obvious changes in either CK2α or -β subunit expression during the time course of the experiment (Fig. 1*B*), indicating that the change in kinase activity could be ascribed to specific activity.

The serum-dependent activation of CK2 suggests a potential role for it in cell cycle progression and a possible association with molecules involved in regulating progression through G_1. Thus, we screened for interacting molecules by analyzing proteins associated with immunoprecipitates of tetrameric CK2 holoenzyme, using cell lysates prepared from cells treated with 10% FBS for 3 h. CK2α and -β were present in the immunoaffinity-purified fraction and were found to interact with several proteins, including eIF5, by in-gel digestion and mass spectrometric analysis (Fig. 1*C*).

Cell Cycle-Dependent Phosphorylation of eIF5. The results described above raise the possibility that eIF5 is a physiological target for phosphorylation by CK2. To determine whether eIF5 is actually

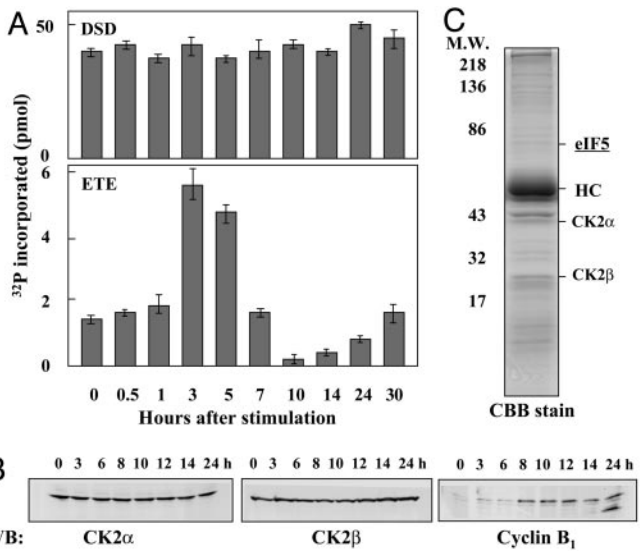

Fig. 1. Activation of CK2 by growth-promoting stimuli. Serum-starved TIG-7 cells were stimulated with 10% FBS and collected at the indicated times after treatment. (*A*) CK2 activity recovered in the immunoprecipitates with anti-CK2α antibody was measured by using two synthetic peptide substrates. Specific activity was calculated by subtracting radioactivity in the absence of peptide substrate. (*B*) Cellular content of CK2α and -β subunits and cyclin B_1. Cells stimulated with FBS for the indicated times were analyzed by immunoblotting with specific antibodies. (*C*) Protein profile of immunoprecipitates with anti-CK2α antibody from TIG cells stimulated with FBS for 3 h. Proteins were subjected to SDS/PAGE and visualized by Coomasie brilliant blue (CBB) staining.

phosphorylated *in vivo*, we prepared extracts from TIG-7 cells metabolically labeled with ^{32}P-orthophosphate and analyzed immunoprecipitated eIF5 for radiolabeling. Lysates were prepared from cells in different cell cycle stages, including serum-starved cells in G_0, cells stimulated with 10% FBS for 3 h in G_1, cells arrested in S phase by double thymidine treatment, and cells arrested in G_2/M by nocodazole treatment. As shown in Fig. 2*A*, the phosphorylation level of eIF5 was low in G_0, dramatically increased by 3 h after FBS treatment, and increased further in S and G_2/M phases. These changes were confirmed by following cells for 24 h after serum stimulation (Fig. 2 *A*, lanes 5–11, and *B*, lanes 1–4). Phosphorylation of eIF5 was induced by 3 h after stimulation, increased further over 10 h, and remained high in cells progressing through S and G_2/M phases. On the other hand, when logarithmically growing cells were serum-starved and gated into G_0/G_1, the eIF5 phosphorylation level decreased to basal levels over 48 h (Fig. 2*C*, lanes 5–9). The cell number was constant for up to 72 h during starvation.

Next, we examined whether the interaction between eIF5 and CK2 occurs in a cell cycle-dependent manner. Immunoprecipitates with anti-eIF5 antibody were prepared from cells at different stages in the cell cycle and analyzed by immunoblotting using an anti-CK2α antibody probe. These experiments demonstrated that eIF5 associated with CK2α only in G_1, and not in G_0 or G_2/M, phases (Fig. 2*C*, lanes 1–4). Time-course experiments also demonstrated that this association was observable 3 h after FBS stimulation, increased in a time-dependent manner until 10 h, and quickly disrupted after 12 h. The highest level of interaction was observed ≈8 h after FBS stimulation (Fig. 2*C*, lanes 5–11).

To determine whether CK2 could be responsible for the phosphorylation of eIF5, we tested the ability of this enzyme to phosphorylate eIF5 *in vitro*. As shown in Fig. 2*D*, GST-eIF5 was readily phosphorylated by recombinant CK2 *in vitro*, and this phosphorylation was markedly reduced in the presence of the CK2 inhibitor, heparin, suggesting that CK2 is the kinase responsible for

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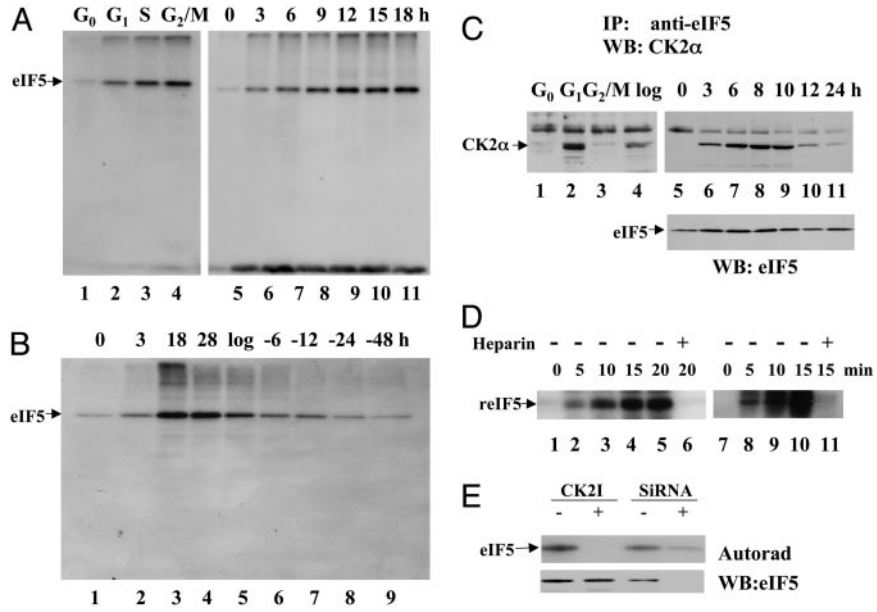

Fig. 2. eIF5 is phosphorylated during cell cycle progression. (*A*) Serum-starved TIG-7 cells were stimulated with 10% FBS for varying time periods and labeled with ^{32}P-orthophosphate for the final 1 h. Cell lysates were prepared for immunoprecipitation with anti-eIF5 antibody. Proteins were separated by SDS/PAGE (12% acrylamide), and radioactive bands were visualized by autoradiography. (*Left*) G_0, unstimulated cells (lane 1); G_1, stimulated for 3 h (lane 2); S, thymidine-arrested (lane 3); and G_2/M, nocodazole-arrested (lane 4). (*Right*) G_0 (lane 5) and cells stimulated with 10% FBS for the various periods indicated (lanes 6–11). (*B*) Phosphorylation levels of eIF5 were associated with growing state. Cell lysates were prepared as in *A* (lanes 1–4), growing nonsynchronized cells (lane 5), and cells starved by lowering the serum concentration to 0.2% for 6–48 h (lanes 6–9). (*C*) Association of eIF5 and CK2α. Immunoprecipitates obtained from cells as in *A* with an anti-eIF5 antibody were separated by SDS/PAGE and blotted with anti-CK2α antibody. (*D*) *In vitro* phosphorylation of eIF5 by CK2. Phosphorylation mixtures containing recombinant eIF5 and CK2$\alpha\beta$ proteins were incubated at 30°C, as indicated, in the absence (lanes 1–5) or presence (lane 6) of 10 ng/ml heparin. The CK2$\alpha\beta$ holoenzyme recovered in immunoprecipitates from growing TIG-7 cells was used in lanes 7–11. All samples were separated by SDS/PAGE and subjected to autoradiography. (*E*) Inhibition of CK2 reduced phosphorylated eIF5 levels. Serum-starved TIG-7 cells were stimulated for 6 h and treated with apigenin (CK2I+), left untreated (−), pretreated with CK2–short interfering RNA (siRNA+) or left untreated (−), then starved and stimulated for 6 h. eIF5 protein was labeled and immunoprecipitated as in *A*. Total lysates were analyzed in parallel for eIF5 protein content (*Lower*).

phosphorylation of eIF5. Similar results were obtained when the recombinant CK2 was substituted with endogenous enzyme that was purified from logarithmically growing TIG-7 cells (Fig. 2*D*, lanes 7–11). Based on these results, we predicted that inhibition of CK2 activity would reduce phosphorylation of eIF5 *in vivo*. TIG-7 cells were treated with the selective CK2 inhibitor apigenin or were pretreated with short interfering RNA to suppress the expression of CK2 and then labeled with ^{32}P-orthophosphate after serum stimulation. As shown in Fig. 2*E*, *in vivo* phosphorylation of eIF5 was attenuated as expected. Thus, both pharmacologic and molecular inhibition of CK2 demonstrates that eIF5 is phosphorylated by CK2.

MS was then used to identify *in vitro* phosphorylation sites in eIF5 by peptide mass analysis and peptide sequencing (tandem MS). Recombinant eIF5 protein was phosphorylated with human CK2$\alpha\beta$ holoenzyme for 20 min, then digested with trypsin and analyzed by QSTAR-TOF MS. We compared the peptides from unphosphorylated and phosphorylated eIF5, searching for peptides that increase in mass by 80 Da. As a result, two phosphorylated serine residues, Ser-389 and -390, were unambiguously identified (Fig. 3*A*). The mass of a fragment ion from the phosphorylated peptide was 160 Da larger than that of the unphosphorylated peptide, corresponding to phosphorylation at both Ser-389 and -390 (Fig. 3*B*). Similar spectra of the phosphoforms of the peptide were observed when *in vivo*-labeled eIF5 was digested for mass spectrometric analysis. Phosphorylation of Thr-207 and -208 in eIF5 was also detected, although these sites were phosphorylated to a lesser extent than the Ser sites.

Inhibition of eIF5 Phosphorylation by Mutation of CK2 Sites. To confirm these phosphorylation sites, a series of site-directed mutants were engineered and confirmed by sequencing. We constructed plasmids for expression of FLAG-tagged WT eIF5, FLAG-tagged eIF5 in which Ser-389 and -390 were substituted by Ala (Ser-389/390-Ala, M1), FLAG-tagged eIF5 in which Thr-207 and -208 were substituted by Ala (Thr-207/208-Ala, M2), and FLAG-tagged eIF5 possessing all four mutations (Ser-389/390-Ala–Thr-207/208-Ala, M3) as shown in Fig. 3*A*. Although the FLAG-tagged WT eIF5 was phosphorylated after 6 h of serum stimulation, only a small proportion of the mutant proteins were phosphorylated under similar expression conditions (Fig. 3*C*, lanes 3–5). Mutants M1 and M3 exhibited much lower levels of phosphorylation than the WT *in vivo*. It is noteworthy that phosphorylation of the endogenous eIF5 protein was suppressed by the expression of eIF5 mutant proteins (Fig. 3*C*, lanes 8–10): in particular, M1 almost completely suppressed phosphorylation of endogenous eIF5, and M2 and M3 had similar effects, although to a lesser extent. These results indicate a dominant-negative effect of the eIF5 mutants on phosphorylation levels of endogenous eIF5 protein and indicate the importance of phosphorylation at Ser-389 and -390 on eIF5.

The β Subunit of CK2 Is Necessary for eIF5 Phosphorylation. As to CK2, it is evident that kinase activity is absolutely dependent on the presence of the regulatory β subunit. The CK2β subunit plays an important role in the assembly of tetrameric CK2 complexes, in enhancing the catalytic activity and stability of CK2, and in modulating the substrate selectivity of CK2. In many cases, it is apparent that CK2β is responsible for the docking and/or recruiting of CK2 substrates or potential regulators. In this respect, potential CK2 targets as well as potential CK2 regulators interact with CK2 via interactions with CK2β.

We analyzed whether phosphorylation levels of eIF5 are affected by CK2β *in vitro* and whether CK2 is the only kinase that phosphorylates eIF5. Phosphorylation mixtures containing recombinant eIF5 and CK2α were incubated for 10 min in the presence of different concentrations of CK2β or of cyclin/CDK6 kinase, PKA, or mitogen-activated protein kinase (MAPK) instead of CK2α. As shown in Fig. 4*A*, phosphorylation of eIF5 increased as the molar ratio of β to α was increased. Also, neither CDK6, PKA, nor MAPK phosphorylated eIF5 under the conditions where CK2α phosphorylates the substrate in the presence of the CK2β subunit. Recombinant mutants of eIF5 in which Ser-389 and -390 were replaced by alanine were also resistant to phosphorylation by CK2 in the presence of CK2β, although the phosphorylation levels of WT eIF5 depended on the concentration of CK2β (Fig. 4*B*). These results suggest that CK2 is the sole kinase acting on eIF5, phosphorylating mainly Ser-389 and -390.

Phosphorytion of eIF5 for Translation Initiation Assembly. eIF5 is involved in the formation of part of a multifactor complex com-

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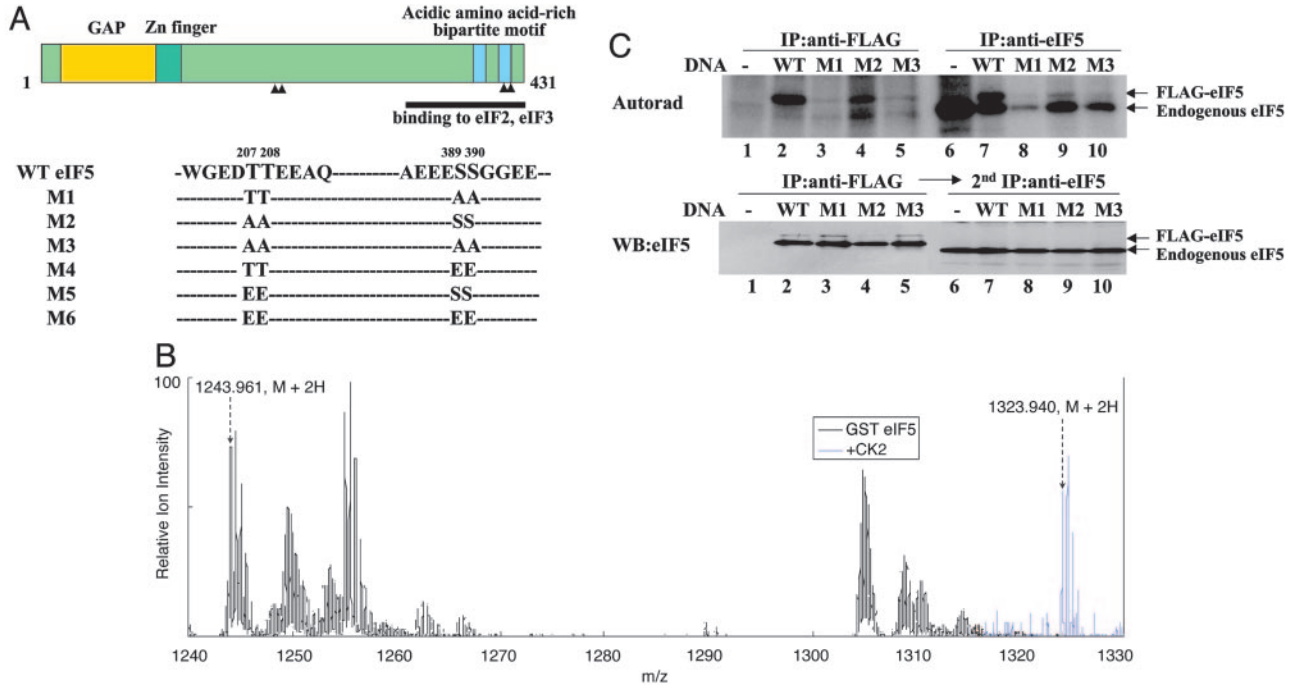

Fig. 3. Phosphorylation sites in eIF5. (*A*) Schematic structure of human eIF5. Phosphorylation sites in the eIF5 protein *in vivo* and *in vitro* by CK2 were determined by mass spectrometric analysis, and locations are indicated by arrowheads. Ser-389 and -390 were identified as major phosphorylation sites, and Thr-207 and -208 were minor sites. WT and mutants of eIF5 used in this study are shown. (*B*) MS analysis of eIF5 phosphorylation sites. QSTAR-TOF mass spectrum of peptides from recombinant eIF5 (black) vs. recombinant eIF5 phosphorylated by CK2 (blue). The shift in peptide mass from 1243.961 to 1323.940 (2H) by +160 Da is shown. The peptide sequence corresponds to residues 384-EAEEESSGGEEEDEDENIEVVY-405 of human eIF5. Both Ser-389 and -390 of eIF5 are phosphorylated by CK2. (*C*) Ala mutations at phosphorylation sites in eIF5 were engineered, and those phosphorylation levels were monitored *in vivo*. COS-7 cells expressing WT or mutant of FLAG-tagged eIF5 were stimulated for 6 h, then labeled as shown in Fig. 2. Mock transfected (lanes 1 and 6), WT (lanes 2 and 7), M1 (lanes 3 and 8), M2 (lanes 4 and 9), and M3 (lanes 5 and 10) were used for immunoprecipitation with anti-FLAG (lanes 1–5) or -eIF5 (lanes 6–10) antibodies. (*Lower*) The amount of immunoprecipitated eIF5 was determined by immunoblotting with anti-eIF5 antibodies.

prising translation–initiation factors including eIF1, eIF2, eIF3, and eIF4 (23, 24). We examined the effect of the CK2-mediated phosphorylation of eIF5 on the formation of this translation–initiation complex. Sucrose gradient-velocity sedimentation was used to fractionate lysates from cells expressing WT or M1 eIF5, and the distribution of eIF2, eIF3, and eIF5 in various fractions was examined. As shown in Fig. 5 *A* and *B*, the expression of M1 eIF5 shifted the distribution of each component of the translation–initiation complex toward the top of the gradient: in WT-expressing cells, the majority of eIF2 and eIF3 was detected in fractions 4 or 5, respectively, coeluting with the ribosomal S6 protein peak, and eIF5 was mainly detected in fractions 5–10. In contrast, eIF3 was recovered in fractions 4 and 5 of M1-expressing cells, and only a trace amount of eIF2 was found in fraction 6, which was behind the peak of eIF3. eIF5 was observed in fractions 7–10 and in lighter fractions (data not shown).

To confirm that reduced phosphorylation of eIF5 disrupts complex formation, eIF5 WT and M1 mutant were probed for interactions with eIF2. FLAG-tagged eIF5 WT and M1 DNA were independently expressed in HEK293 cells, which were then analyzed in an attempt to identify interactions of these proteins with eIF2. As shown in Fig. 5*C Upper*, the eIF5 mutant associated poorly with endogenous eIF2 in the anti-FLAG immunoprecipitates when compared with WT eIF5. In addition, the recombinant eIF5 mutant disrupted the interaction between WT eIF5 and eIF2 *in vitro* (Fig. 5*C Lower*). These results suggest that the phosphorylation of eIF5 by CK2 is important for the proper association of eIF5 with the mature translation–initiation complex, at least with eIF2, which is engaged in translation *in vivo*.

FCS Measurements of eIF5. Next, we analyzed how the M1 mutation affects the association of eIF5 with the translational machinery by using FCS. This method measures fluctuation of fluorescent molecules in a subfemtoliter confocal volume at very high time resolution (25). By calculating the autocorrelation function of such fluctuating fluorescence signals, complex formation with relatively immobile molecules can be detected in living cells. Because eIF5 forms a large complex with other members of eIF as well as ribosomes, and the current results predict that CK2 phosphorylation of eIF5 is required for functional complex formation, we reasoned that the diffusion profile of mutant eIF5 should be distinct from that of the WT molecule.

In Fig. 5*D*, our measurements of both the GFP-tagged eIF5 WT and M1 are summarized. The average autocorrelation curve clearly indicates that the M1 mutant diffused in a less-restricted manner than the WT. Fitting of the WT eIF5 diffusion profile to the theoretical multicomponent diffusion models revealed the presence of two types of slow components other than the fast simple diffusional component; one is in the order of milliseconds and the other, in the order of seconds (Fig. 5*E* and Fig. 6, which is published as supporting information on the PNAS web site). It is likely that the millisecond diffusion fraction represents the molecules associated with other eIFs or translation complexes tethered to membranes, because diffusion in this range is typical of membrane proteins. In the M1 autocorrelation curve, the slowest fraction was not detected, and the millisecond diffusion was markedly reduced. Consistent with this, GFP-eIF5 WT showed large deviation from the one-compartment model, whereas the M1 curve moderately resembled the one-component model, although the two-compartment model was best-fitted (Fig. 5*E*). This indicates that GFP-eIF5 WT requires CK2 phosphorylation sites to interact with the relatively immobile molecular machinery such as the putative initiation complexes on ribosomes.

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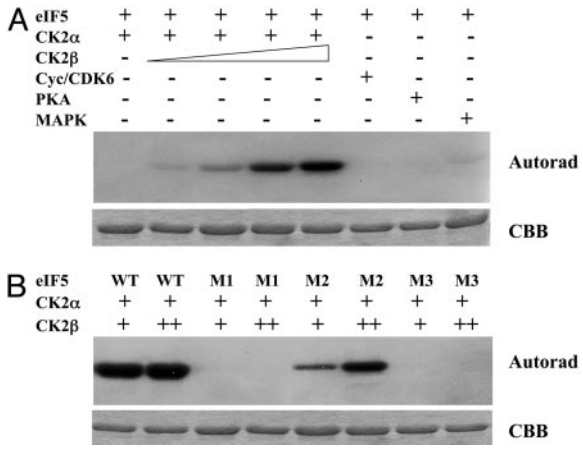

Fig. 4. The β subunit of CK2 is necessary for eIF5 phosphorylation. (*A*) Analysis of eIF5 phosphorylation by CK2 *in vitro*. Phosphorylation mixtures containing recombinant eIF5 were incubated at 30°C for 10 min in the presence of CK2α at molar ratio of β/α of 0, 0.1, 0.2, 0.5, 1.0, respectively, or in the presence of cyclin/CDK6 kinase, PKA, or mitogen-activated protein kinase, and then separated by SDS/PAGE and autoradiographed as in Fig. 2. (*B*) Mutation of Ser-389 and -390 to alanine in eIF5 reduced phosphorylation by CK2. The molar ratio of CK2β to α is 0.5 (+) and 1.0 (++), respectively. Recombinant eIF5 protein in the phosphorylation reactions is shown (*Lower*).

Impairment of Cell Cycle Progression and Growth in eIF5 Mutants. The above results suggest that eIF5 mutants may affect translation initiation and cell proliferation. Therefore, we monitored COS-7 cells expressing FLAG-eIF5 mutants by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays. Cell proliferation was significantly abrogated by all three mutants and reached 30% reduction in M1- compared with WT eIF5-expressing cells. In contrast, when using eIF5 mutants in which the phosphorylated threonine and serine residues were substituted with glutamic acids, cell proliferation was enhanced by 30–50% in M4-, M5-, or M6-expressing cells that mimic phosphorylated states in eIF5 (Fig. 7*A*, which is published as supporting information on the PNAS web site). DNA histogram analysis was carried out to quantify populations in G_0/G_1, S, and G_2/M stages, and the expression of M1 was also associated with a marked decrease in cell populations in the G_2/M phase and an increase in the G_0/G_1 and S phases (Fig. 7*B*).

Because cyclin B_1 is an important regulator of G_2/M transition (26), we determined its expression level in HEK293 cells that were transfected with WT or M1 eIF5. For reference, we also analyzed cells that were transfected with the CK2α-subunit or APC protein C-terminal fragment (APC-C) that is a negative regulator of CK2 activity (15). M1 expression reduced cyclin B_1 levels by ≈40%. APC-C expression also reduced levels by ≈30%, whereas WT eIF5 and CK2α had no effect on the content of cyclin B_1. Also, cyclin B_1-asociated kinase activity was significantly inactivated by M1 or APC-C expression (Fig. 8, which is published as supporting information on the PNAS web site). This verifies that blocking phosphorylation of eIF5 by site-directed mutagenesis inhibits cell cycle progression.

Discussion

Although activation of CK2 in response to stimuli has been reported in a variety of cell types, definitive evidence that this kinase is regulated in response to growth factor stimuli has been difficult to reproduce (14). In this study, we successfully demonstrated a dramatic and reproducible enhancement of CK2 activity in response to FBS by using extracts from synchronized TIG-7 cells. CK2 was activated 5-fold by 3 h after stimulation and returned to basal levels 7 h later. Because CK2 has been considered to be constitutively active and not regulated by second messengers, mechanisms for enhancement of its activity in cells includes gene expression, covalent modifications such as phosphorylation, and interactions with other cellular molecules. The results presented in Fig. 1 show no change in CK2α or -β levels over the 24 h after FBS stimulation. The results in Fig. 2 demonstrate no change in CK2α and -β subunit phosphorylation in immunoprecipitates with eIF5. However, in-

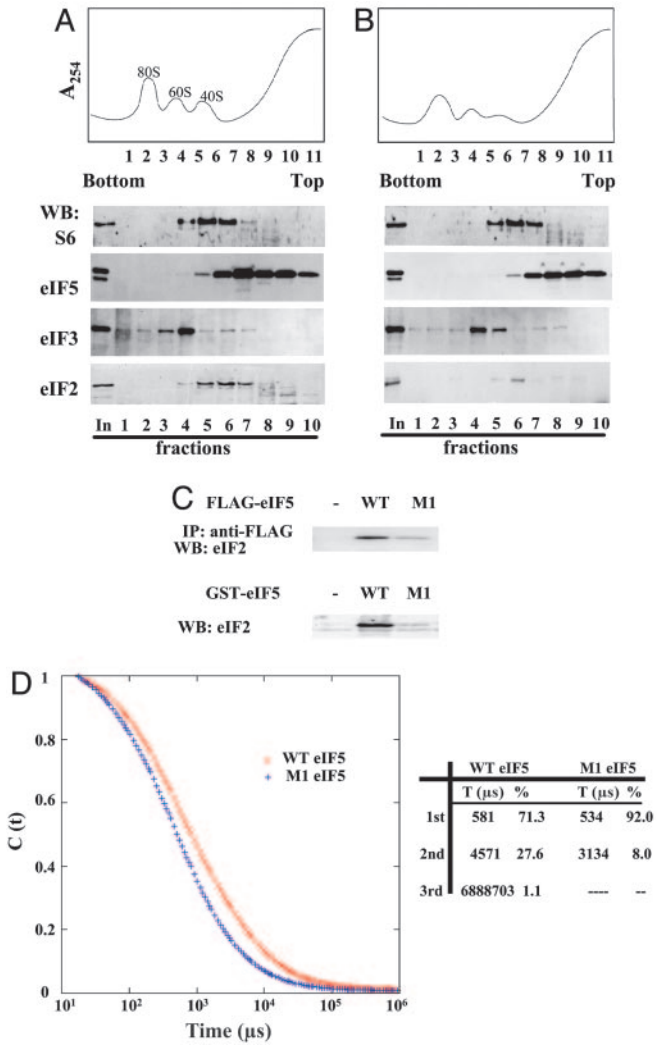

Fig. 5. Analysis of the translation initiation protein complex profile of WT and mutant eIF5-expressing cells. Cell lysates were prepared from exponentially growing cultures of HEK293 cells transfected with either WT (*A*) or M1 (*B*) FLAG-eIF5, and each sample (≈2 mg of protein) was separated on a 5–40% (wt/vol) sucrose gradient. Absorbance at 254 nm was measured. Anti-FLAG agarose beads were added to each eluate to separate the FLAG protein, then immunoprecipitates were analyzed for eIF2, eIF3, and FLAG-eIF5 by immunoblotting by using specific antibodies. The anti-S6 antibody blots are shown in parallel to estimate the 40S–43S ribosome fractions. Representatives of five separate experiments are shown. (*C*) eIF5 mutant disrupt interaction with eIF2. (*Upper*) FLAG-eIF5 WT or M1 was expressed in HEK293 cells and immunoprecipitated by using anti-FLAG agarose resin. (*Lower*) GST-eIF5 WT or M1 proteins were incubated separately with HEK293 cell lysates for 4 h at 4°C and pulled down by glutathione-Sepharose resin. Proteins were separated and visualized by anti-eIF2. (*D*) FCS measurements of GFP-eIF WT and M1. COS7 cells were transfected with pEGFP-eIF5 and incubated for 3 h at 37°C. Sixty-five spots in the cytoplasm were chosen for the measurements, and the fluorescence was recorded for 45 s by FCS. Of these spots, 47 (WT) or 60 (M1) were used for calculating the average autocorrelation function. The normalized autocorrelation is plotted for GFP-eIF5 WT (red) and M1 (blue). The diffusion time and fraction of each component were estimated by fitting to three- (WT) or two-component (M1) models.

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creased recovery of CK2α in immunoprecipitates with eIF5 was evident by at least 10 h after stimulation (Fig. 2). These observations imply that the activity of CK2 is controlled by interaction with regulatory molecules rather than by protein expression or direct phosphorylation. In this context, our previous results demonstrating that the tumor suppressor protein APC associates with CK2 and suppresses its activity in a cell cycle-dependent manner are relevant (15), although the explicit mechanism of the molecular interaction is still unclear.

In this study, we demonstrate that CK2 interacts with and phosphorylates eIF5, a key molecule for the formation of the translation initiation complex. The association is observed when CK2 activity is elevated by growth factor stimulation. Phosphorylation sites in eIF5 catalyzed by CK2 were identified, and mutants lacking these sites were verified to eliminate phosphorylation by CK2. We demonstrated that phosphorylation of Thr-207 and -208 occurs to a lesser extent than that of Ser-389 and -390. The presence of acidic residues on both sides of Thr-207 and -208 may evoke the possible involvement of phosphorylation at these sites for efficient phosphorylation at Ser-389 and -390. Furthermore, these mutants exhibited dominant-negative effects on phosphorylation of endogenous eIF5. Based on these results, we conclude that eIF5 is an intracellular target for phosphorylation by CK2.

eIF5 plays a critical role in initiation of protein synthesis (23). The premature complex mediates hydrolysis of GTP on eIF2, leading to release of eIF2-GDP and eIF3 from the 40S subunit, which is essential for the subsequent joining of the 60S ribosomal subunit with the 40S complex to form an elongation-competent 80S initiation complex. Our data show that the transition from the 40S to the 80S translation initiation complex is impaired by overexpression of the M1 mutant, indicating an important role for CK2-mediated phosphorylation of eIF5 on the maturation of the complex. Based on these results, we hypothesize that CK2-mediated phosphorylation of eIF5 is required for its association with eIF2 and its function as a GTPase-activating protein for eIF2. A role for eIF5 in the maturation of the complex has been supported by findings in yeast. eIF5 binds to eIF3-NIP1 and eIF2β through the AA-box domain at the C terminus of eIF5 to modulate close interaction and deletion of 7–12 amino acids from the AA-boxes, leads to destruction of the multifactor complex, and diminishes the fraction of ribosomes engaged in translation *in vivo* (20, 27, 28). Because the CK2-mediated phosphorylation sites Ser-389 and -390 are located in the AA-box (Fig. 3*A*), it is reasonable to suggest that phosphorylation within the AA-box regulates interaction with other initiation factors.

In accordance with the above data, results of FCS measurements indicate that the WT protein was immobilized by associating with a large protein complex. In contrast, random diffusion of the GFP-eIF5 mutant in a real-time manner revealed significant prolongation, compared with that of the WT. This indicates that GFP-eIF5 WT has a significant interaction with the putative translation complex through CK2 phosphorylation sites, thus random diffusion of the eIF5 mutant is increased when the latter lacks these sites.

The phosphorylation level of eIF5 was found to be quite low in serum-starved quiescent cells and dramatically increased by growth stimuli. This high level was maintained during progression of the cell cycle, even in periods when CK2 activity was suppressed and the two molecules were dissociated. Eliminating growth factors from the culture medium was sufficient to reduce the phosphorylation levels of eIF5. This discrepancy should be clarified in future studies.

It is noteworthy that CK2-mediated phosphorylation of eIF5 might be responsible for cell cycle progression. We demonstrate that removal of CK2 phosphorylation sites in eIF5 attenuates cell cycle progression and causes reduced S to M transition. In particular, expression of M1 leads to an obvious reduction in the cell population in M phase and increases the population in G_1 and S phases. These results indicate a mechanism in which eIF5 phosphorylation by CK2 is needed for normal cell cycle progression at the S/G_2 boundary. Indeed, the expression level of cyclin B_1, and eventually its associated kinase activity that are essential for entry into mitosis, was reduced by M1 eIF5 mutant expression (Fig. 8). A similar reduction in cyclin B_1 levels was observed in cells expressing APC protein C-terminal fragment protein, an endogenous CK2 inhibitor. Therefore, we conclude that CK2-dependent phosphorylation of eIF5 is required to assemble the normal translation initiation complex, which may stimulate the translation of cell cycle regulators such as cyclin B_1, promoting synthesis of cellular proteins needed for mitotic entry. Notably, this effect is specific, because no significant changes in expression were observed for cyclin A, D, or E (data not shown). In this context, eIF5 phosphorylation by CK2 might be an important modifier for the progression of cell cycle from S to G_2/M phase by reflecting translation status.

Collectively, these data suggest that CK2 may participate in the regulation of proteins that have important functions associated with cell cycle progression. We propose a model in which CK2-mediated eIF5 phosphorylation constitutes an important molecular event for the progression of the cell cycle.

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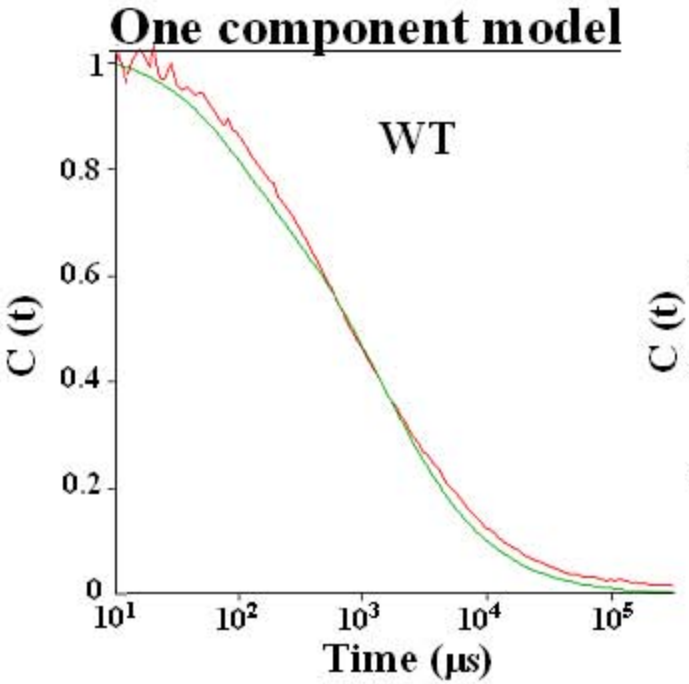
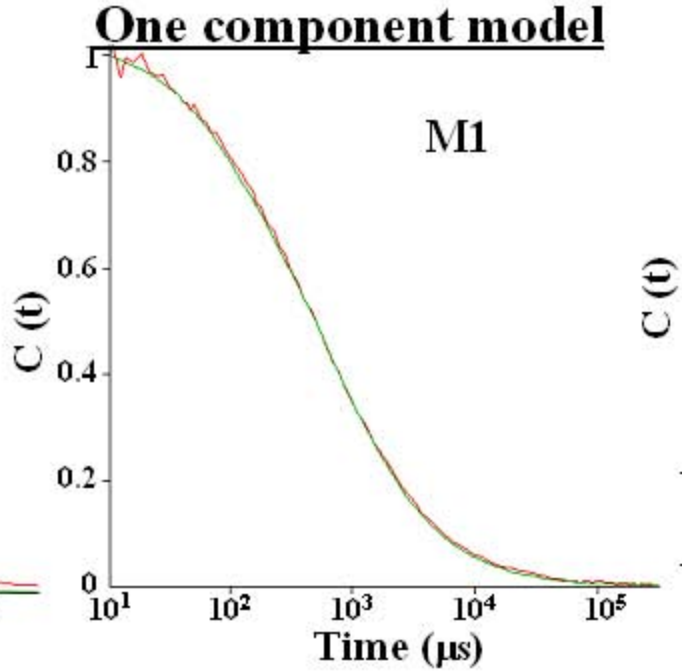
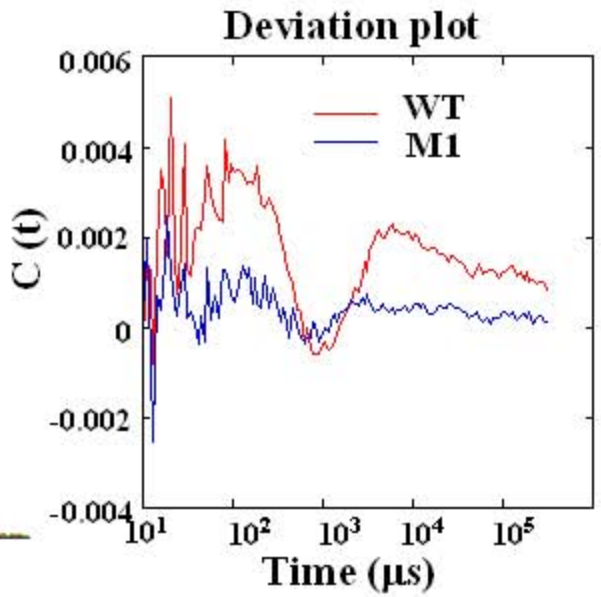
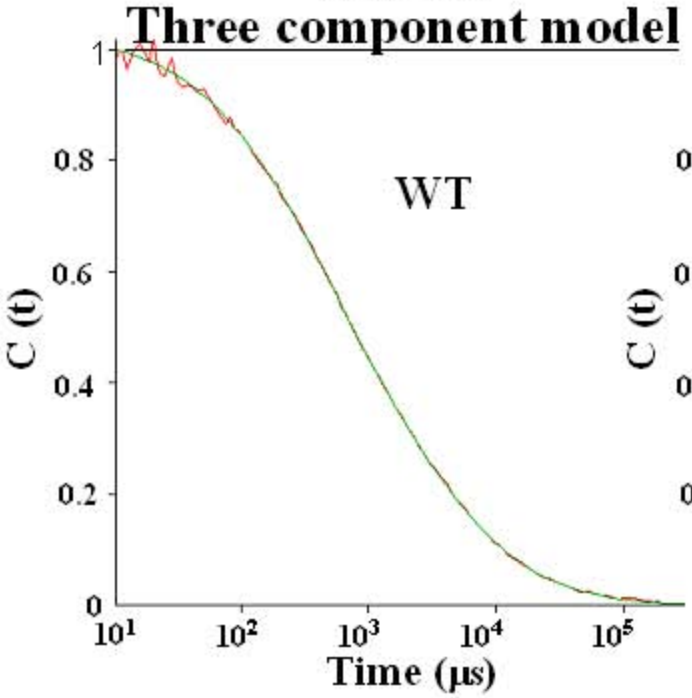
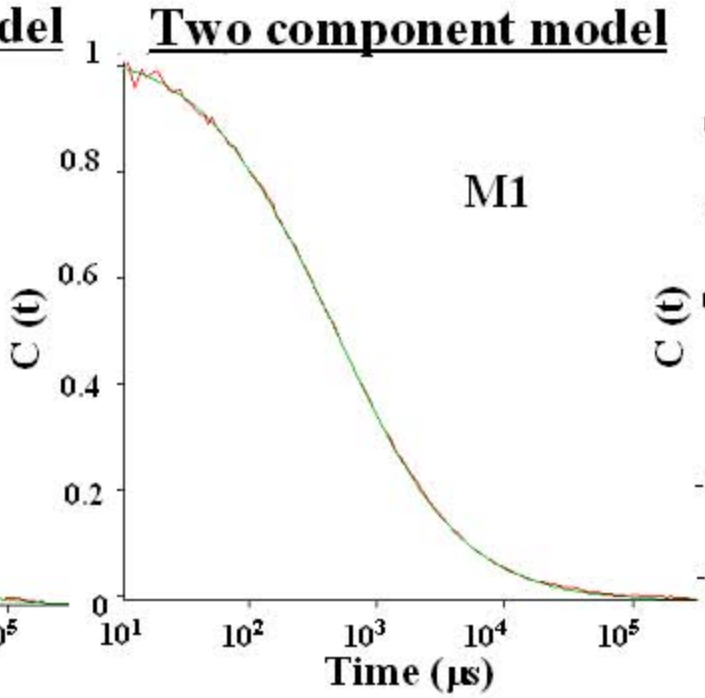
Fig5E. Homma

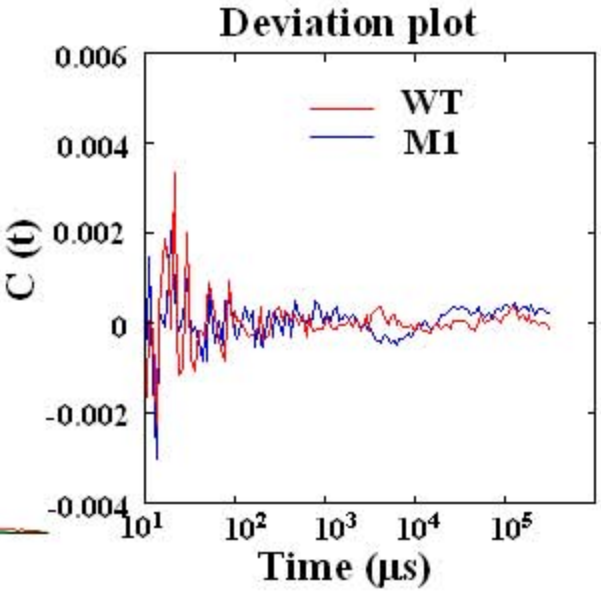

One component model
WT
C (t)
Time (μs)

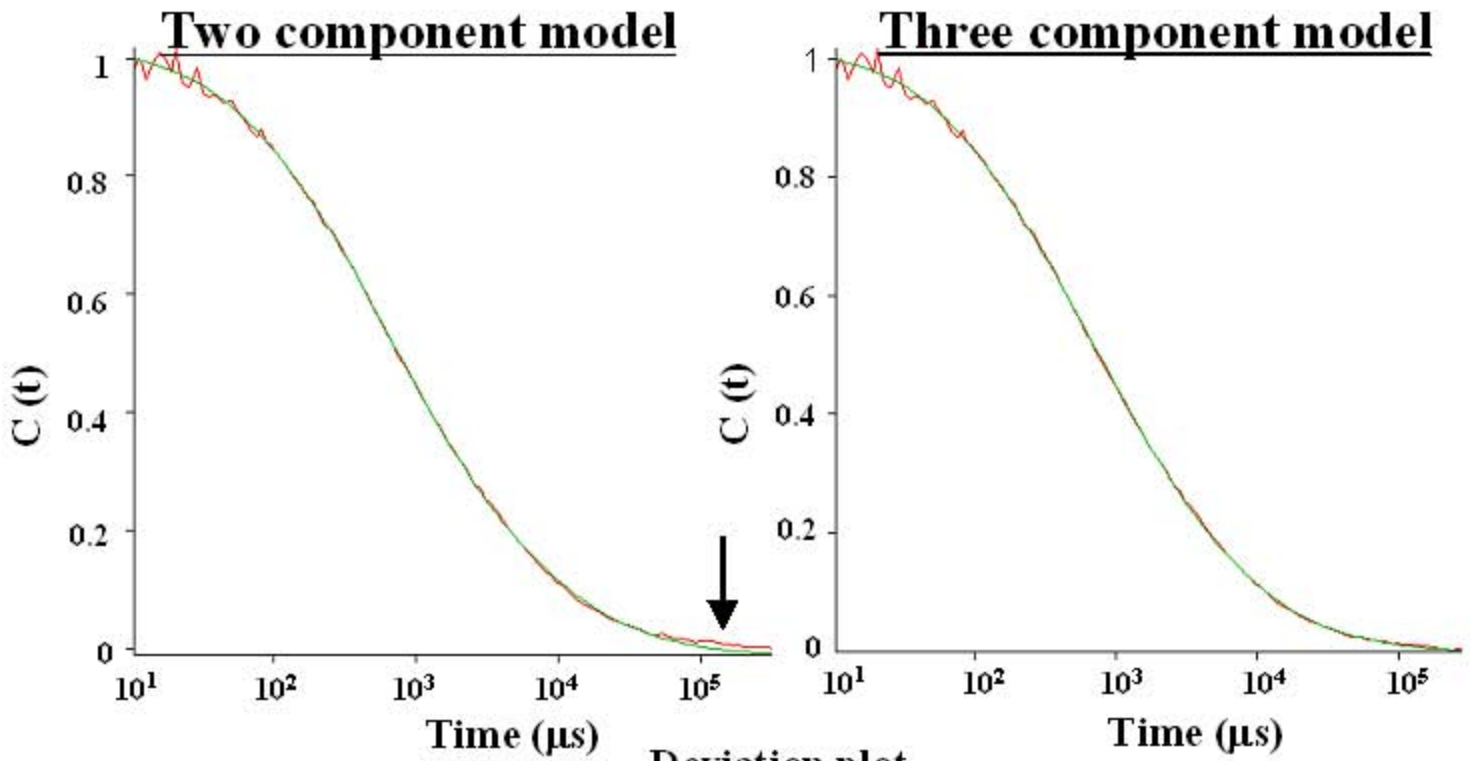

One component model
M1
C (t)
Time (μs)


Deviation plot
WT
M1
C (t)
Time (μs)


Three component model
WT
C (t)
Time (μs)


Two component model
M1
C (t)
Time (μs)


Deviation plot
WT
M1
C (t)
Time (μs)


Two component model
Three component model
C (t)
Time (µs)
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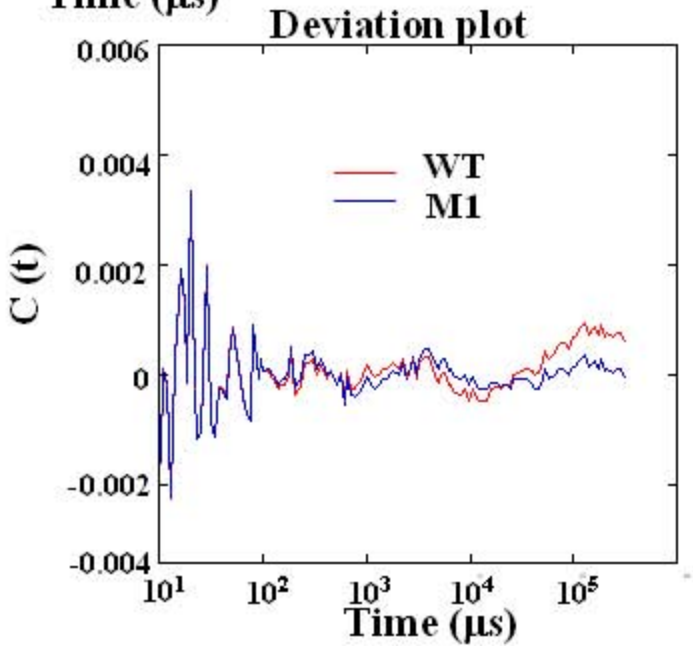
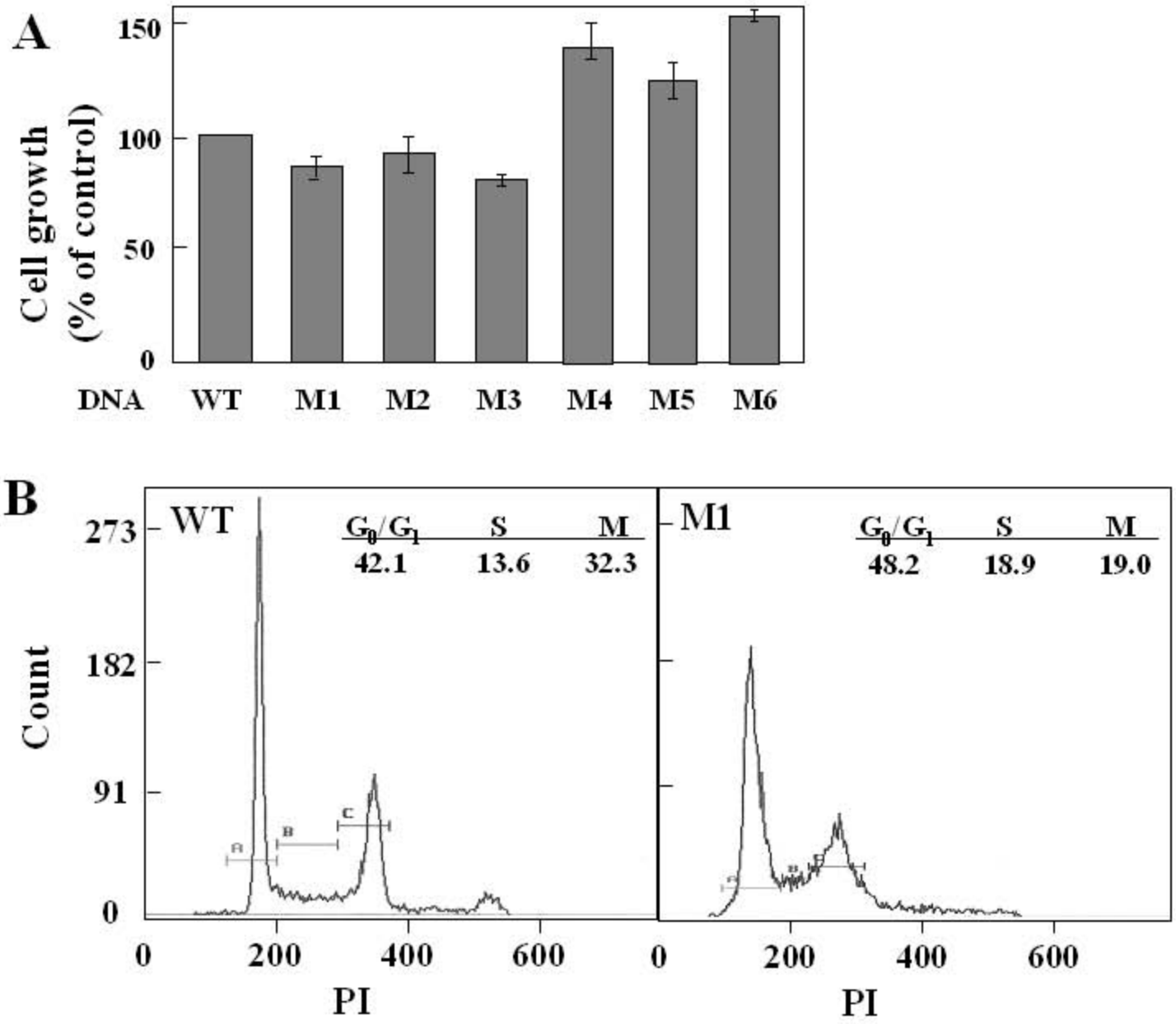
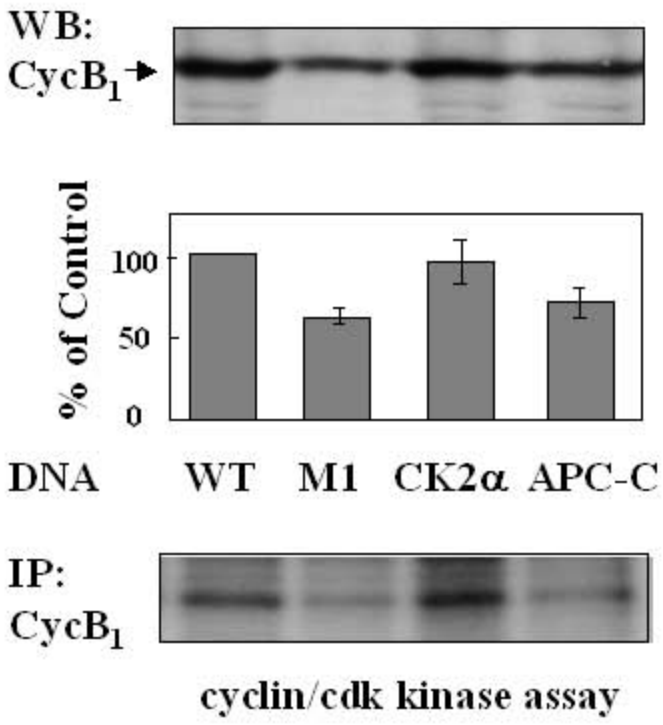
0.8
0.6
0.4
0.2
0
10^1
10^2
10^3
10^4
10^5


Deviation plot
WT
M1
C (t)
Time (µs)
0.006
0.004
0.002
0
-0.002
-0.004
10^1
10^2
10^3
10^4
10^5


A
Cell growth
(% of control)
150
100
50
0
DNA
WT
M1
M2
M3
M4
M5
M6
B
WT
M1
G0/G1
S
M
42.1
13.6
32.3
48.2
18.9
19.0
Count
273
182
91
0
A
B
C
0
200
400
600
PI


WB:
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% of Control
100
50
0
DNA
WT
M1
CK2α
APC-C
IP:
CycB1
cyclin/cdk kinase assay